DIOSCIN INDUCES APOPTOSIS IN BREAST CANCER CELL LINES THROUGH MITOCHONDRIAL SIGNALING PATHWAY

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Abstract
Dioscin, a natural steroid glucoside, has been broadly investigated. However, its anti-cancer activities on human breast cancer cells are still not clear. In this study, we investigated the effect of Dioscin on the activities of Caspase 3 and 9 and also the expression of anti-apoptotic proteins BAX and BCL-2 were examined. The activities of Caspase 3 and 9 in Dioscin-treated groups were significantly increased compared with control group. Western blotting results showed that Dioscin significantly down regulated the expressions of BCL-2 and upregulated the expressions of BAX. This results indicate that the Dioscin has anticancer activities against human breast cancer cells through activating mitochondrial signaling pathway. Moreover, in silico analysis of proteins such as Caspase 3, Caspase 9, BAX and BCL-2 were analyzed. The molecular docking results showed that the Dioscin has good binding affinity with all the proteins with significant docking score.

Keywords: Dioscin, Breast cancer and Apoptosis.

Introduction
Breast cancer comprises ~30% of new cancer diagnoses, and is one of the most common causes of cancer mortality in women (1). In spite of many advances in cancer research, breast cancer remains a serious problem and denotes a high biomedical research precedence. Moreover, recent research interest focus on breast cancer arising in young women. Numerous research studies suggested that in women aged below 45 years, it is undeniably the foremost cause of cancer associated deaths. And to date with available data also stated breast cancer in young women accounts as a major problem in developing countries perversely to developed countries and this type of cancer vanished an unpredictable number of young women life every year (2). It is restricted by surgery, chemotherapy, radiotherapy, and frequently supported by hormonotherapy (3).

Apoptosis is a naturally occurring process of programmed cell death. In general, drug-induced apoptosis is one major reason for treatment of cancer, and some signal pathways are involved in the process (4). Among them, apoptosis in mitochondria is the best known pathway. Mitochondria plays a central role in cell apoptosis, since both the intrinsic and extrinsic apoptosis pathways can converge at the mitochondrial level and trigger the alteration of mitochondrial membrane permeability (5). Mitochondria-mediated apoptosis is mainly in the aspects including release of apoptosis active substance, activation of Bel-2 family members (6) and downstream Caspase family proteins (7). Since many cancer cells have mutations in key apoptotic genes and defects in caspase signaling, cancer cells become resistant to traditional chemotherapy drugs, which become a major limitation in treating cancer. An induction of apoptosis in tumor cells has long been recognized as the essential approach for cancer therapy. However, defects in apoptosis can promote drug resistance of some tumor cells to apoptosis, which is an important clinical problem.

Traditional medicines have been used to treat cancer because of their high efficiency and low toxicity. Some natural products have been widely used as the anti-cancer agents for treatment of cancers and
thus exploration of effective natural products from traditional medicines for treatment of breast cancer is reasonable. Dioscin is a potent inducer of apoptosis in various cancer cell lines. Several studies have shown that dioscin induces death receptor and mitochondria-mediated apoptosis in different cancer cell types via alteration of expression of the mitochondria-associated proteins, cell cycle regulatory proteins, death receptors or death ligands, via increasing caspases activities, and acting as a potent multidrug resistance reversal agent (8,9). However, the molecular mechanism by which Dioscin initiates apoptosis in human breast cancer cell lines has not been well characterized. Hence the purpose of the study is planned to investigate the underlying mechanism of Dioscin induced apoptosis on human breast cancer cell lines.

**Materials and Methods**

**Measurement of caspase 3 and 9 activities:**
The Breast Cancer cells were plated in 6-well plates (2 × 10^5 cells/well) and Dioscin (2, 4 and 6 µM) were treated for 24 h. The activities of caspase 3 and 9 were measured using caspase activity assay kits (Beyotime, Shanghai). The p-nitroaniline (pNA) release was quantified by measuring OD405 value using a microplate reader (Thermo, USA).

**Western blot analysis**
Protein expression was assessed for BAX, BCL-2 (protein of interest) and β-Actin (internal controls) in Breast Cancer cell lines by western blot method. Proteins were extracted from cells and separated on polyacrylamide gel on the basis of size and transferred on to PVDF membranes. Following transfer, the proteins of interest were detected by incubation of the membrane with specific antibodies, followed by detection with an enzymatically labelled (HRP-conjugated) secondary antibody. The protein expression was visualized by chemiluminescent method using enhanced chemiluminescence (ECL) reagent.

**Molecular Docking:**
The chemical structure of Dioscin was obtained from the PubChem (https://pubchem.ncbi.nlm.nih.gov/), saved in its SDF format, and converted to the mol2 format by Discovery Studio 3.0. The PDB IDs of the candidates BAX and BCL-2 were derived from the UniProt database, and the corresponding protein three-dimensional structure was obtained from the RCSB PDB (http://www.rcsb.org/) database and saved in PDB format. The ligand and protein were energy minimized and prepared for docking using Dockprep tool in UCSF chimera software. Molecular docking was performed using Autodock Tools-1.5.6, and the docking score was used to assess the binding affinity of the target to the Dioscin molecule. The two and three dimensional plan of the docking results was presented by Discovery Studio 2019 Client.

**Results and Discussion**
In order to demonstrate the molecular mechanism of Dioscin induced apoptosis of breast cancer cell lines, measurement of caspase 3 & 9 activities and the expression of BAX (anti-apoptotic protein) & BCL-2 (pro-apoptotic) proteins were determined in human breast cancer cell lines. We reported that Dioscin decreased the viability of MCF-7 and MDA-MB-231 in a dose dependent manner with a half maximal inhibitory concentration (IC_{50}) of 7.5 µM and 8 µM respectively (10).

MCF-7 and MDA-MB-231 cell lines were treated with 2, 4 and 6 µM concentrations of Dioscin for 24 h. The activity of caspase 3 and 9 was enhanced by Dioscin treatment (Figure 1). Compared to the control, the activity of caspase 3 and 9 was increased at concentration dependent manner. Compared to the control group, the expression of BCL-2 were suppressed, while the expression of BAX were increased when concentrations of Dioscin were increased (Figure 2).

Molecular docking approaches have used to examine whether the Dioscin firmly bind with breast targets such as Caspase 3, 9, BAX and BCL-2. The three dimensional structure of four different proteins were subjected molecular docking approach at the desired grid coordinates of the protein...
molecules with the help of Lamarckian Genetic Algorithm (GA) of AutoDock. The test compound Dioscin showed good binding affinity with all the targets with good docking score.

Figure 3 depicted that interaction of Dioscin with human Caspase 3 (PDB code: 3GP0). It shows significant docking score (-8.6 kcal/mol), the visual inspection of molecular interaction of Dioscin with active site of Caspase 3, clearly indicating that the test compound was seated firmly at the binding pocket (Figure 3), and the intermolecular interactions such as conventional hydrogen bond, carbon hydrogen bond, Alkyl and pi-Alkyl bond formation with caspase 3 were have depicted in Figure 3. The hydroxyl group of Dioscin from two hydrogen bond interactions with Asn73, and the oxygen atom of keto-ring of Dioscin form a hydrogen bond interaction with Lys224, and also with Lys 242 of B chain of Caspase 3. In addition to the conventional hydrogen bond, the test compound Dioscin also make three and two pi-Alkyl bonds with Phe247, and Phe250 respectively. Furthermore the residue Glu246 form van der Waals interaction with caspase 3 and these intermolecular interactions shows that the all these interactions may stabilizing the protein-ligand complex structure.

In Figure 4, it was depicted that intermolecular interaction of Dioscin with Caspase 9. From the figure 4 It was noticed that the Dioscin shows finest docking score (-10.4 kcal/mol) compared to other three apoptotic regulatory protein targets. Dioscin form five conventional hydrogen bond interactions with residues such as Gly277, Asp340, Ser339, As148 and Leu335 via oxygen and hydroxyl group of Dioscin. In addition to the conventional hydrogen bond interaction it also forms and 9 Alkyl interactions with residues such as Ala141, Pro336, Leu145, and Ala141 (table 1). The stable hydrogen bond interactions and other alkyl interactions may responsible for the firmly attachment of Dioscin in the binding site of the caspase 9 and provide high binding affinity with the protein.

The docking score of BAX (8.6 kcal/mol) (Figure 5) is more or less similar to that of BCL-2 (-8.8 kcal/mol), and form single strong hydrogen bond interaction with Ala96 via carbon atom of Dioscin and one pi-sigman bond interaction with Phe114 via oxygen atom of aromatic ring of the Dioscin. Furthermore it forms 12 alkyl and pi-alkyl interaction with residues such as Phe105, Leu59, Leu63, Val111, Val110 (4) and Leu113, Ala97 and Phe100 (2), which indicating that Dioscin shows more binding affinity with BAX protein via interaction of important active site residues.

BCL-2 is an important anti-apoptotic protein paly major role in inhibition of apoptosis in cancer cell. The interaction of Dioscin with anti-apoptotic protein BCL-2 was evolved via molecular docking studies. Figure 6 depicted the interaction of Dioscin with BCL-2 protein and it was noticed that hydroxyl group of the test compound form hydrogen bond interactions with residues such as Glu165, and Trp30 and oxygen atom form two hydrogen interactions with Ser167. In addition it also forms two hydrogen bond interactions with Gly33 and Glu29 via carbon atom of the test compound. Furthermore it forms 7 alkyl and pi-alkyl interactions with residues like Pro168, Val162 (2), Tyr28 (2), and Arg26 (2). From the molecular interaction studies it was concluded that the test compound Dioscin shows more binding affinity and number of hydrogen and other interactions with all the four apoptotic regulator proteins and these in silico studies highly correlates our in vitro gene expression and protein expression analysis in breast cancer cells. This study clearly indicated that the test compound Dioscin significantly acts as either antagonist or agonist against apoptotic regulator proteins, hence it may acts as a potential adjuvant in the cancer treatment.

Conclusion
In conclusion, this study demonstrated that Dioscin regulates apoptosis in breast cancer cells through mitochondria-mediated. We suggest that the use of Dioscin may be used as a drug for enhancing apoptosis and increasing the anticancer potential.

References

**Figures and Tables**

**Figure 1:** Effects of Dioscin on the activities of caspase 3 and 9 on MCF-7 and MDA-MB-231. Data are presented as mean ± SD. Significant at *P<0.05, **P<0.01 compared to control cells.
Figure 2: Dioscin-induced modulation of BAX and BCL-2 proteins on MCF-7 and MDA-MB-231. Relative changes in protein bands were measured using densitometric analysis with the control being 1.0-fold. Significant at *P<0.05, **P<0.01 compared to control cells.

Figure 3: Docked complex of Caspase 3 with Dioscin.

Figure 4: Docked complex of Caspase 9 with Dioscin.

Figure 5: Docked complex of BAX with Dioscin.
Figure 6: Docked complex of BCL-2 with Dioscin.

Table 1: Molecular interaction of Dioscin with Four different target proteins with docking score

<table>
<thead>
<tr>
<th>S.No</th>
<th>Receptors</th>
<th>Docking score</th>
<th>Types of interaction</th>
<th>No. of interaction</th>
<th>Interaction residues</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Caspase 3</td>
<td>-8.6 kcal/mol</td>
<td>Conventional hydrogen bond, Carbone hydrogen bond, Pi-Alkyl bond</td>
<td>5</td>
<td>Glu246, Asn73 (2), Lys224, Gly33, Arg26, Glu29, Pro168, Val162 (2), Try28 (2), Arg26 (2)</td>
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<tr>
<td>3</td>
<td>BAX</td>
<td>-8.8 kcal/mol</td>
<td>Carbone hydrogen bond, Akyl and Pi-Alkyl bond, Pi-sigma bond</td>
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<td>Ala96, Phe105, Leu59, Leu63, Val111, Val110 (4), Leu113, Ala97 and Phe100 (2) Phe114</td>
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<tr>
<td>4</td>
<td>BCL-2</td>
<td>-8.2 kcal/mol</td>
<td>Conventional hydrogen bond, Carbone hydrogen bond, Akyl and Pi-Alkyl bond</td>
<td>4</td>
<td>Trp30, Ser167 (2), Glu165 Gly33, Arg26, Glu29 Pro168, Val162 (2), Try28 (2), Arg26 (2)</td>
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